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PAPER

Insight into hydrolytic reaction of *N*-acetylated L-histidylglycine dipeptide with novel mechlorethamine platinum(II) complex. NMR and DFT study of the hydrolytic reaction

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The reaction of K_2PtCl_4 with the alkylating agent mechlorethamine hydrochloride, at a molar ratio of 1 : 2, results in the formation of 2-chloro-*N*-(2-chloroethyl)-*N*-methylethylammonium-tetrachloridoplatinate(II) complex. The hydrolytic activity of the novel Pt(II) complex was tested in the reaction with *N*-acetylated L-histidylglycine dipeptide at a molar ratio 1 : 1. It was shown that the hydrolytic reaction, performed at 60 °C in acidic medium, leads to the regioselective cleavage of the amide bond involving the carboxylic group of histidine. Density functional theory was used to explore the structures of the proposed participants in the hydrolytic reaction.

Introduction

Alkylating agents are highly reactive compounds. Many of them are synthetic drugs. These compounds react with many electron-rich atoms to form covalent bonds. Nucleophilic groups, such as amino, phosphate, carboxyl, sulfhydryl or imidazolyl moieties in proteins, as well as nucleic acids, can be alkylated. The chemotherapeutic role of alkylating agents derives from their ability to interfere with genetic (DNA) material, giving mono-adducts and cross-links. Mechlorethamine ($CH_3N(C_2H_4Cl)_2$, code-name HN2) is one of the first non-hormonal chemotherapy drugs known as nitrogen mustard.[1] It is a bifunctional agent with two 2-chloroethyl moieties which react covalently with the adjacent guanine residues in each strand of DNA, building a bridge between the DNA strands. The preferred site of alkylation on DNA is the N7 position of the imidazolyl moiety of guanine.

On the other hand, cisplatin ($Pt(NH_3)_2Cl_2$), the most widely used anticancer drug at present, and its analogues (for example carboplatin) are classified as non-classical alkylating agents, or alkylating-like agents, regarding their principle function of binding to DNA. They are used for the treatment of testicular, ovarian, head, neck and lung cancer.[2–4] However, their clinical usefulness has been limited by side effects, such as nephrotoxicity, neurotoxicity and ototoxicity, and by the emergence of cancer cells resistant to cisplatin.[5–7] Therefore, other similar platinum complexes play an important role in the development of anticancer drugs. For all these complexes and their analogues binding occurs to the N7 position of the imidazole ring of the purine bases of DNA. Furthermore, it has been found that some Pt(II) and palladium(II) complexes can behave as artificial metallopeptidases and promising reagents for the cleavage of unreactive amide bonds of peptides and proteins.[8–13] It is known that the peptide bond is extremely unreactive and that the half-life for its hydrolysis in neutral solution is several hundred years at room temperature and pH 4–8.[14,15] Hydrolytic cleavage of proteins, however, plays functional and regulatory roles in physiological processes, such as control of the cell cycle, transcription, antigen processing, and apoptosis. Selective proteolysis can be achieved with enzymes and synthetic reagents. Several proteolytic enzymes are used for cleavage, but application of enzymes is limited by their special requirements for temperature and pH. Since uncatalyzed hydrolysis of peptides is extremely slow, relatively fast methods of artificial cleavage are needed. Transition-metal complexes of Pd and Pt are promising agents for the hydrolytic cleavage of peptides and proteins.[16–24] However, considering the clinical usefulness of Pt(II) complexes and the great affinity of Pt(II) complex ions for the nitrogen of heterocycles (for example the imidazole ring of histidine), it is surprising that such interactions have not been extensively investigated for peptides comprising of sulfur-containing amino acids (for example methionine). Namely, studies on the coordination behavior of Pt(II) complexes to histidine-containing peptides are scarcely reported and, also, the mechanism of the hydrolytic reaction of peptides in the presence of Pt(II) promoters has not been completely elucidated. For the clarification of this mechanism, it was shown to be necessary to investigate these regioselective cleavage reactions with structurally different Pt(II) complexes.

In this paper we report the synthesis, spectral characterization and hydrolytic properties of the novel 2-chloro-*N*-(2-chloroethyl)-*N*-methylethylammonium-tetrachloridoplatinate(II) complex ($[H2N2]_2[PtCl_4]$). Since the new complex contains HN2 as a classical alkylating agent, and Pt(II) ion, as a non-classical alkylating agent, it was interesting to study its reaction with imidazole-containing dipeptide. Moreover, in order to mimic

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endopeptidases, a hydrolytic reaction of the complex with *N*-acetylated L-histidylglycine dipeptide (AcHis-Gly) was carried out. This reaction was studied by using ^{1}H NMR spectroscopy and density functional theory, in order to examine the structures of the proposed reaction participants.

Experimental

The compounds D_2O and K_2PtCl_4, were obtained from Aldrich Chemical Co. All common chemicals were of reagent grade. Mechlorethamine hydrochloride (HN2×HCl), dipeptide L-histidylglycine (His-Gly), were obtained from Sigma Chemical Co. The terminal amino group in His-Gly was acetylated by a standard method to obtain AcHis-Gly.[17] The IR spectra in the solid state were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer using the KBr pellet technique. Elemental microanalysis for carbon, hydrogen, and nitrogen was done at the Faculty of Chemistry, University of Belgrade. All pH measurements were made at 25 °C. The pH meter (Iskra MA 5704) was calibrated with Fischer certified buffer solutions of pH 4.00 and 7.00. The results were not corrected for the deuterium isotope effect. Reactions of AcHis-Gly with Pt(II) complex in D_2O solutions were followed by ^{1}H NMR spectroscopy using a Varian 200 MHz spectrometer. The Pt(II) complex and the peptide were mixed in an NMR tube at a molar ratio 1 : 1. The starting pH was 6, whereas its final value was 4. All reactions were carried out at 60 °C. The internal reference was TMS.

Computational method

All calculations were conducted using Gaussian09[25] with the M06 hybrid functional.[26] The triple split valence basis set 6-311++G(d,p) was used for C, H, O, N, and Cl,[27] whereas LANL2DZ+ECP[28] was employed for the Pt center. Geometrical parameters of all investigated species were optimized in water, using the CPCM model (Polarizable Conductor Calculation Model, ε = 78.36). Vibrational analysis was performed for all structures. All calculated structures were verified to be local minima (all positive eigenvalues) for ground state structures by frequency calculations. The experimental and simulated IR spectra of the Pt(II) complex were compared. As expected, the computed vibrational frequencies were overestimated. Due to the lack of the scaling factor for the applied theoretical model, the calculated frequencies were decreased by 5%, and agreement with the experimental values was achieved. The natural bond orbital analysis[29] (Gaussian NBO version) was performed for all structures. The ^{1}H NMR properties of the Pt(II) complex and crucial reaction product were predicted, and the chemical shifts for all hydrogen atoms relative to TMS were calculated. For the simulation of the ^{1}H NMR spectrum, the model mentioned above, which involves different basis sets for nonmetals and the Pt center, was not suitable. For this reason, the M06/LANL2DZ method was used for the prediction of the ^{1}H NMR properties.

Synthesis of $[H2N2]_2[PtCl_4]$

The $[H2N2]_2[PtCl_4]$ complex was synthesized from K_2PtCl_4 and 2 equivalents of HN2×HCl. In the course of 3 h, the reaction of 0.41 g (1 mmol) of K_2PtCl_4 dissolved in 15 cm^3 of water with 0.384 g (2 mmol) of HN2×HCl, at 50–60 °C, afforded a reddish-brown solution which was left at room temperature for two days. The precipitated light reddish-brown crystals were filtered off, washed with ethanol, air-dried and showed a melting point of 122–124 °C. Yield 0.684 g (95%). Calculated for $[H2N2]_2[PtCl_4] = C_{10}H_{24}Cl_8N_2Pt$ (FW = 651.02): C, 18.45; N, 4.30; H, 3.72%; found: C, 17.53; N, 3.95; H, 3.45%.

Spectral characterization of the $[H2N2]_2[PtCl_4]$ complex: ^{1}H NMR spectrum (200 MHz, D_2O): δ = 3.04 (3H, CH_3-NHR_2, s), 3.71 (4H, -CH_2-Cl, t, J = 5.0 Hz), 4.02 (4H, -CH_2-NHR_2, t, J = 5.0 Hz) ppm; IR (KBr): ν = 299, 670, 739, 933, 1117, 1463, 2758, 3018.3 cm^{-1}.

Table 1 Selected bond distances (Å) in the reactants

Complex	
Pt–Cl (all)	2.39
Cl–H	2.35
Cl–H	2.48
N–H (both)	1.04
Dipeptide	
C–O	1.23
C–N	1.33

Results and Discussion

Structure examination of the $[H2N2]_2[PtCl_4]$ complex and AcHis-Gly dipeptide

The structures of the reactants in the hydrolysis reaction, the $[H2N2]_2[PtCl_4]$ complex and AcHis-Gly, were examined using density functional theory. The optimized structures are presented in Fig. 1 and the selected bond distances are given in Table 1.

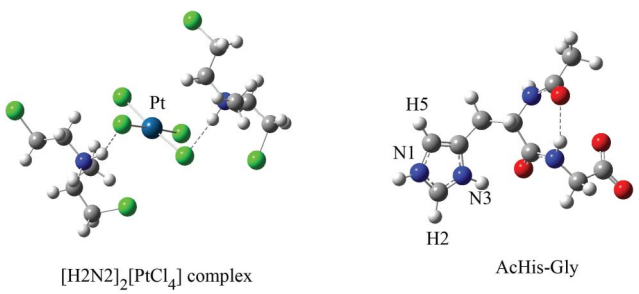

Fig. 1 Optimized structures of the reactants.

The complex $[PtCl_4]^{2-}$ anion exhibits square planar coordination, where the four chlorine anions lie in the equatorial plane. In addition, the two protonated mechlorethamine cations form hydrogen bonding with chlorido ligands. The NBO analysis of the $[H2N2]_2[PtCl_4]$ complex shows that Pt bears only four electron pairs in the d orbitals, thus indicating that this compound is a Lewis acid. The characteristic calculated vibrational frequencies of the complex were as follows: ν = 287 (Pt–Cl), 650 (C–Cl), 743 (C–Cl), 931 (C–N), 1085 (C–N), 1469 (N–H), and 3044 (N–H) cm^{-1}. The calculated chemical shifts for the complex are as follows: δ = 3.10 (CH_3–NHR_2), 3.03 (–CH_2–Cl), and 3.85 (–CH_2–NHR_2) ppm. There is a good agreement between the experimental and computed IR spectra of the complex. As for the simulated NMR spectrum, the only noticeable deviation from the experimental value is that for the protons from

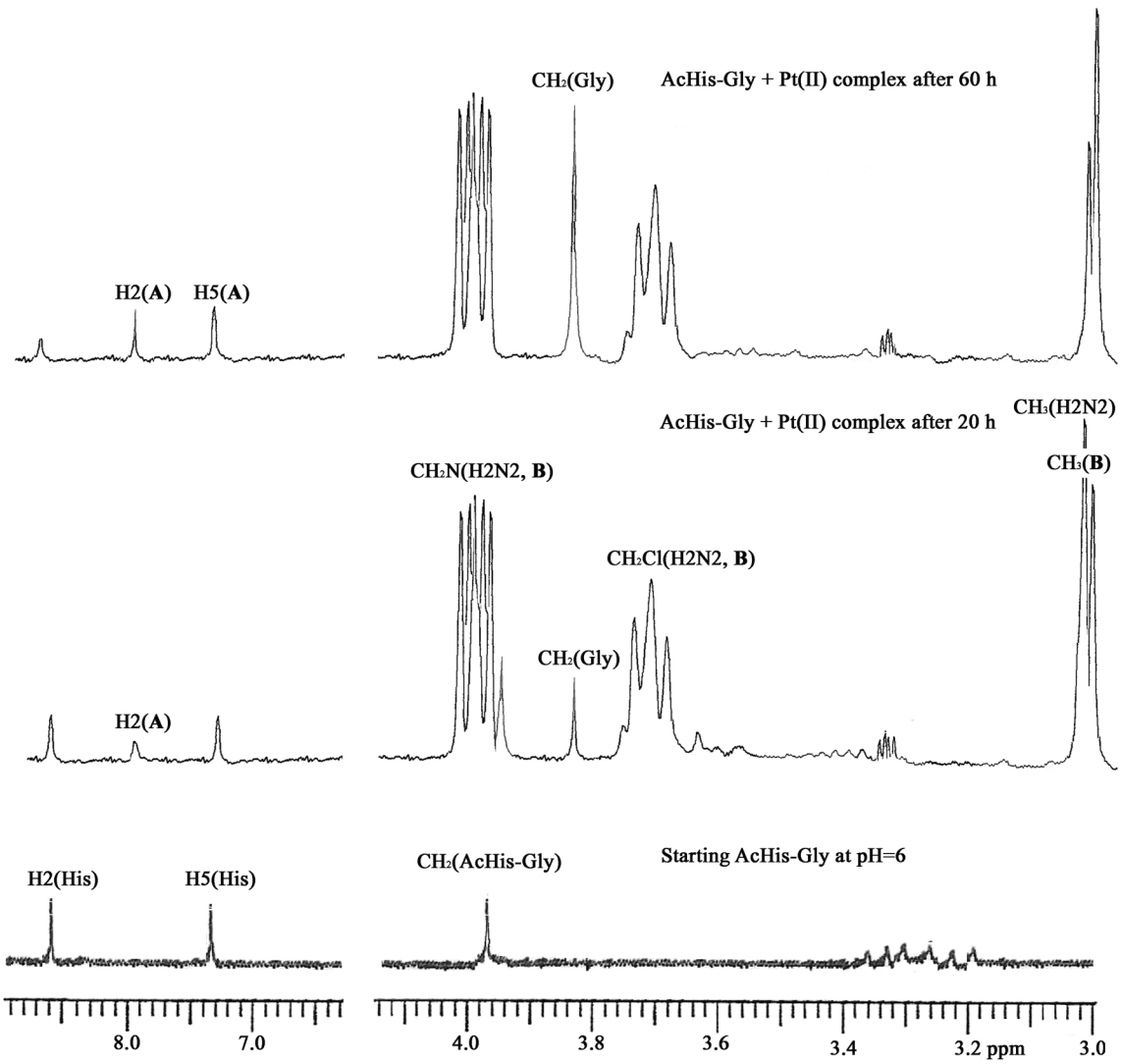

Fig. 2 Parts of ^{1}H NMR spectra for the hydrolytic reaction of AcHis-Gly with $[H2N2]_2[PtCl_4]$ complex as a function of time, in D_2O as solvent. The chemical shifts are given in ppm relative to TMS.

–CH_2– bonded to chlorine. This deviation can be attributed to the fact that the computer simulation does not take into account mutual interactions (strong hydrogen bonds) among the complex molecules. In Fig. 1 the dipeptide is presented as a zwitterion, since the starting pH of the reaction mixture was 6.

Hydrolytic reaction of the $[H2N2]_2[PtCl_4]$ complex with AcHis-Gly

In order to test the novel mechlorethamine Pt(II) complex as an artificial metallopeptidase, a hydrolytic reaction of the complex with AcHis-Gly dipeptide in acidic solution at 60 °C was performed. The Pt(II) complex and dipeptide were mixed in equimolar amounts, with a 20 mM total concentration of both reactants, in D_2O solution. The reaction was monitored by ^{1}H NMR spectroscopy, which proved to be a very useful tool for studying complex enzyme-like hydrolytic reactions. The reaction products were distinguished on the basis of the chemical shifts of the methyl group protons from the mechlorethamine unit of the complex, two imidazolyl protons (H2 and H5), and methylene glycine protons (from non-hydrolyzed substrate and free glycine), Fig. 2. The appearance of a new singlet in the ^{1}H NMR spectrum at 3.814 ppm indicated that the hydrolytic reaction had occurred. During the reaction between the Pt(II) complex and AcHis-Gly, the resonance at 3.96 ppm of methylene glycine protons from the non-hydrolyzed dipeptide decreased, while the singlet at 3.814 ppm for methylene protons of free glycine was increasing. Beyond the reaction time of 60 h, the intensity of the singlet at 3.814 ppm remained unchanged. In addition, a new singlet at 3.02 ppm and a multiplet at 3.97 ppm appeared in the NMR spectrum as indicators that free glycine reacts with the mechlorethamine unit of the complex, yielding the salt **B** ($[C_7H_{17}O_2N_2Cl_2]^+$, Fig. 3). Indeed, the same compound was formed in a separate reaction, where equimolar amounts of mechlorethamine and amino acid glycine were mixed and heated for 60 h at 60 °C.

During the hydrolytic reaction, beside two existing singlets at 7.35 ppm and 8.62 ppm of the two imidazolyl protons H2 and H5 of the starting dipeptide, one new singlet at 7.95 ppm appeared. We found that this peak can be assigned to the H2

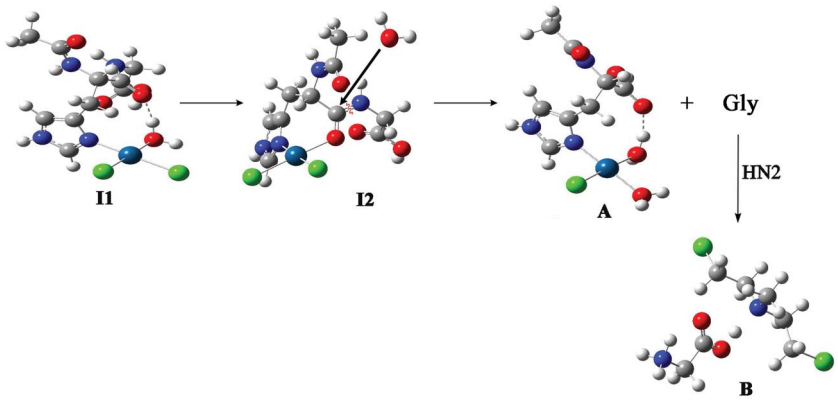

Fig. 3 Optimized structures of intermediates and products.

of the platinated AcHis (**A** in Fig. 3), which was formed as a result of the regioselective cleavage of the amide bond. It is worth pointing out that the final pH value of the reaction mixture was 4 (due to the liberated HCl from the starting complex). Under these acidic conditions, free acetic acid was not detected by NMR spectroscopy, confirming that the reaction is regioselective. Since this hydrolytic reaction was not suitable for kinetic investigation, we used density functional theory in order to analyze its possible mechanism and structures of the intermediates and reaction products. Taking into account that hydrolysis of the complex $[PtCl_4]^{2-}$ in diluted acidic aqueous medium results in the formation of $[PtCl_2(H_2O)_2]$,[30,31] and the fact that N3 and N1 nitrogen atoms of the imidazole moiety are good metal-binding sites in reactions with histidine-containing peptides,[32–37] we assume slow coordination of the protonated dipeptide to the Pt(II), *via* imidazole N3, giving intermediate **I1** (Fig. 3). The Pt(II)-anchored peptide complex, obtained in this way, contains water molecule as a ligand. Since water is a good leaving group, its departure allows the required close approach of the Pt(II) ion to the scissile amide bond. This process leads to coordination of amide oxygen to the Pt(II) and formation of the hydrolytically active intermediate **I2**.[32] Due to this coordination the carbonyl group becomes more polarized (the NBO charges on the oxygen and carbon of the carbonyl group are –0.58, and 0.72) and more active toward external nucleophilic attack of solvent water. In this way regioselective cleavage of the amide bond, involving the carboxylic group of histidine, is achieved, and glycine is liberated. Appearance of the signal at 3.814 ppm in the ^{1}H NMR spectrum (Fig. 2), is in agreement with our proposed model. Our finding is supported with other experiments with different histidine-containing peptides and different Pt(II) complexes, which showed that coordination of the N3 atom of imidazole to the Pt(II) ion affects hydrolytic cleavage of the peptide bond. This was explained through the fact that this coordination mode provides the necessary approach of the Pt(II) ion and its aqua ligand to the scissile peptide bond.[32,35] As a result of the hydrolytic reactions of intermediate **I2** (regioselective cleveage of the amide bond and substitution of the chlorido ligand with water molecule) the complex **A** is formed. The signals at 7.95 and 7.32 ppm in the ^{1}H NMR spectrum can be assigned to H2 and H5 of this compound. The calculated chemical shifts for H2 and H5 are 8.21 and 7.36 ppm. Such agreement between the experimental and calculated NMR spectra of the complex confirms the structure of the reaction product. The NBO analysis of the product **A** shows that Pt forms covalent bonds with all ligating atoms, where the lone electron pairs in the p orbitals of ligating atoms participate with more than 80% in the bonds around platinum. Each Pt σ bonding orbital delocalizes into the *trans* σ^* antibonding Pt orbital. The selected bond distances in **I2**, **A**, and **B** are given in Table 2. Our investigation shows that the free energies of solvation of reactants and products amount to 398 and 487 kJ mol^{-1}, respectively. It can be estimated that the investigated reaction is endothermic, which is in agreement with experimental conditions.

Table 2 Selected bond distances (Å) in the crucial intermediate and products

I2	
Pt–N3	2.04
Pt–Cl	2.35
Pt–Cl	2.37
Pt–O (carbonyl)	2.13
C–O (carbonyl)	1.25
C–N (peptide bond)	1.34
A	
Pt–N3	2.00
Pt–Cl	2.40
Pt–O (H_2O) (both)	2.09
B	
N–H	1.59
O–H	1.04

The mechanism proposed in this work is quite similar to the mechanism of hydrolysis of peptide bonds catalyzed by zinc metalloenzyme carboxypeptidase.[38–43] Taking into account this fact, and the finding that the investigated hydrolytic reaction is regioselective, the novel Pt(II) complex can be considered as a potentional artificial metallopeptidase.

Conclusions

The hydrolytic reaction of AcHis-Gly with the novel $[H2N2]_2[PtCl_4]$ complex was performed under mild reaction conditions. The investigated complex contains two alkylating agents: classical mechlorethamine, and non-classical Pt^{2+} ion. Our results show that in this reaction the anionic part of the complex $[PtCl_4]^{2-}$ is a more potent alkylating agent than the classical mechlorethamine. Since the reaction medium of the investigated hydrolysis was acidic, there were no conditions for significant

formation of reactive aziridinium ion of mechlorethamine, which would react (after nucleophilic ring opening) with imidazole of the dipeptide. Instead, under these reaction conditions, Pt^{2+} reacted as an alkylating agent, yielding the Pt(II)-anchored hydrolytically active complex. It is worth pointing out that this complex is necessary for regioselective cleavage of the peptide amide bond, involving the carboxylic group of histidine.

It was established that the novel Pt(II) complex described here is a very useful compound, due to its alkylating properties and to its ability to act as an artificial metallopeptidase.

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